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Quantifying the combined effect of salt and temperature on the growth of *Listeria* strains isolated from salmon and salmon processing environments

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Abstract

The effects of temperature and salt concentration on the growth of relevant strains of *L. monocytogenes* and *Listeria innocua*, were quantified based on growth in a liquid broth medium, monitored by measuring turbidity. Absorbance curves were used to estimate maximum growth rate and lag time. The growth rates increase with increasing temperatures. The levels were quite similar for both species, although *L. monocytogenes* showed higher growth rate than *L. innocua* at 4ºC. Lag time was more affected both by temperature and salt concentration, and these effects were most prominent for *L. innocua*.

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Keywords: *Listeria*; turbidity; modelling; salt; temperature

1. Introduction

Listeria monocytogenes remains a challenging pathogenic organism for the seafood industry. Due to its ubiquitous presence, potential to contaminate products after processing, and ability to multiply at temperatures as low as 0ºC [1], it is a target for control in many ready-to-eat (RTE) foods, including seafood products [2]. Smoked salmon is produced by salting, smoking, trimming and slicing of fillets. Salting is normally carried out by dry salting or injection salting [3].

Producers of RTE foods are advised to document that the maximum content of *L. monocytogenes* should not exceed 100 CFU/g at the end of the given shelf life period [4-6]. This implies that both the inactivation of potential *Listeria* present on the product as well as the growth limitations of potential

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survivors are critical factors to ensure food safety. The effect of salt on the growth dynamics of potential pathogens like *L. monocytogenes* is of importance to exposure assessment. The growth probability of *L. monocytogenes* has been found to be comparable in cooked salmon and in liquid media (TSB) with relevant levels of salt and phenol added, but only at temperatures higher than 12ºC [7]. The same study also showed that the growth of *L. monocytogenes* was more profoundly affected by salt content and storage temperature, than by phenol content.

In recent guidelines for shelf life determination of RTE-products, documentation of growth potential of *L. monocytogenes* is recommended to be obtained either through actual challenge studies, or through modelling [4]. In this context the applicability and precision of growth models for *L. monocytogenes* is of vital importance for food safety. Generating data for model building based on culturing *Listeria* in the relevant food products is one approach. Less labour intensive is to grow bacteria in growth media and monitor their growth responses through measurements of optical density [8]. Validated growth models have been published for *L. monocytogenes* in seafood products [9-11]. Given the slow growth response of *Listeria* at refrigerated storage temperatures, good models for estimation of growth parameters from optical measurements is still of interest for screening, e.g., strains and new preservation conditions.

The objective of this work is to study the effect of temperature and salt concentration on the growth of *Listeria.* The strains investigated originate from or are relevant for salmon processing. The current study focuses on *Listeria* growth in a liquid medium model system.

2. Material and Methods

2.1. Strains

L. monocytogenes strains (5, 11, 14, 15, 21, 26, 44, 51-2) isolated from salmon and salmon processing environment were obtained from the National Institute of Nutrition and Seafood Research (Norway). Their origin and description are shown in table 1.

Table 1. Origin and description of *Listeria monocytogenes* strains used in this study.

Origin	Factory	Code
Fish, ungutted	A	1BR5
Fish, to be "gravad"	A	1BR26
Fish, swab of fish at filleting line	A	2BR14
Fish, gutted fish on gutting line	B	3BS44
Environment, table for deheading	A	1BR11
Environment, slicing machine in slicing room	A	1BR56
Environment, forceps for picking bones	A	2BR21
Environment, conveyor belt manual gutting line	C	2HF15
Environment, surface sorting box after grader	B	3BS51-2

Sequencing of 16S identified the strains as foodborne L. monocytogenes, serotype 1/2a, and Multiple Loci VNTR analysis divided the strains further into 8 different subtypes. In addition and due to their relevance as model organisms [12;13], two L. innocua strains, ATCC 33090 and CCUG 35613 (= ATCC 51742) were included in the experiments and investigated with respect to their growth kinetic properties.

The cultures were stored frozen at -80ºC in cryovials (Microbank, Pro-Lab Diagnostics, Canada). Before each experiment a frozen bead was recovered in Tryptic Soy Broth (Oxoid, Basingstoke, UK) with 0.6% yeast extract (Merck, Darmstadt, Germany) (TSBYE, 10 ml) at 37ºC over night.

2.2. Bioscreen-experiments

Each overnight recovered suspension was inoculated in a 100 mL erlenmeyer flask containing 25 ml TSBYE and grown to stationary phase (growth conditions: 20h/30ºC/150 rpm). 10-fold serial dilutions of the inoculum were prepared in TSBYE with 0, 2.5 and 5 % (w/v) NaCl. 5 dilutions (10^{-1} to 10^{-5}) of each strain were transferred to 100 well microtiter plates (300 µL in each well). The microtiter plates were mounted in a Bioscreen C (Oy Growth Curves Ab Ltd., Helsinki, Finland) programmed to measure absorbance at 600 nm (abs_{600nm}) at regular time intervals. Prior to each measurement, the plates were shaken for 10 seconds (default setting). The temperatures investigated were 4, 12 and 30ºC. When experiments were conducted at sub ambient temperatures, the Bioscreen C was placed in an incubator (ICP 600, Memmert GmbH + Co. KG, Schwabach, Germany) with temperature setting close to the set-point temperature of the Bioscreen.

For each strain a calibration curve was set up to relate turbidity measurements (abs_{600nm}) to total viable cell counts (TVC) on Tryptic Soy Agar (Oxoid, Basingstoke, UK) with 0.6% yeast extract (Merck, Darmstadt, Germany) (TSAYE). This calibration curve was used to determine the initial cell concentration (N_i), and the cell concentration at abs_{600nm} = 0.2 (N_{turb}). The growth parameters, i.e., the maximum specific growth rate, μ_{max}, and the lag time, λ, were estimated from turbidity measurement data ($abs_{600\ nm}$) by determination of the time to detection (TTD) as shown in Figure 1, and by using the serial dilution method [14;15] (1):

$$TTD_i = \lambda + \frac{\ln(N_{turb} / N_i)}{\mu_{\max}} \qquad (1)$$

TTD_i is the time to detection, i.e., the time it takes for the $abs_{600\ nm}$ to reach 0.2. λ is the lag time, μ_{max} is the maximum growth rate, N_{turb} equals the TVC at $abs_{600\ nm}$ = 0.2, and N_i is the initial TVC in the well. Four dilutions were used to determine each value. In most cases results were obtained using the average of 2 parallel wells in 3 independent replicate runs.

3. Results and Discussion

Some of the parameters required for the estimations are shown in an actual Bioscreen growth curve graph (*L. innocua* ATCC 33090 in TSBYE + 5% NaCl at 30º C) in Fig. 1.

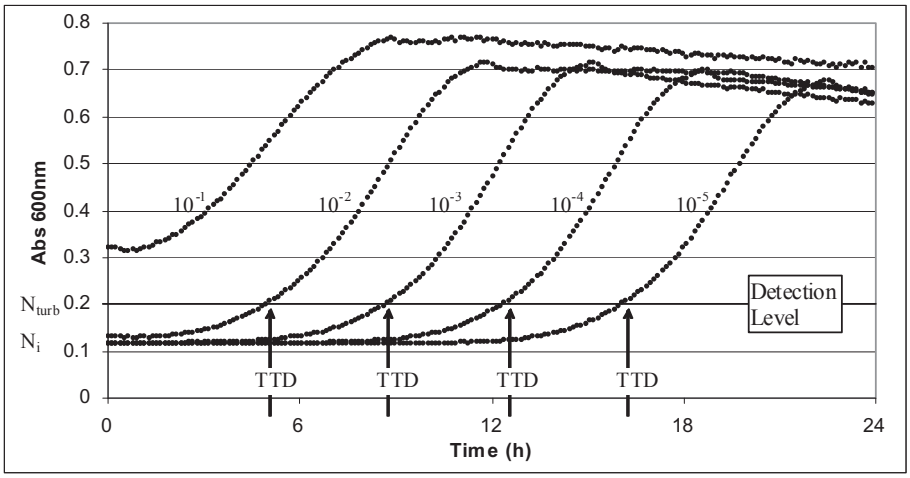

Fig. 1. Turbidity growth curves *L. innocua* ATCC 33090 in TSBYE + 5% NaCl at 30º C, obtained at Abs = 600 nm.The Time to Detection (TTD) is indicated for each of 5 decimal dilutions

From equation (1), λ and μ_{max} could be determined by plotting $\ln(N_{turb}/N_i)$ vs. TTD, as illustrated in Figure 2 for *L. innocua* ATCC 33090 in TSBYE + 5% NaCl at 30° C (as above). Based on the regression line equation (y = 1.6323x + 2.6177), λ and μ_{max}, were estimated to be 2.6 and (1/1.6323) = 0.61, respectively.

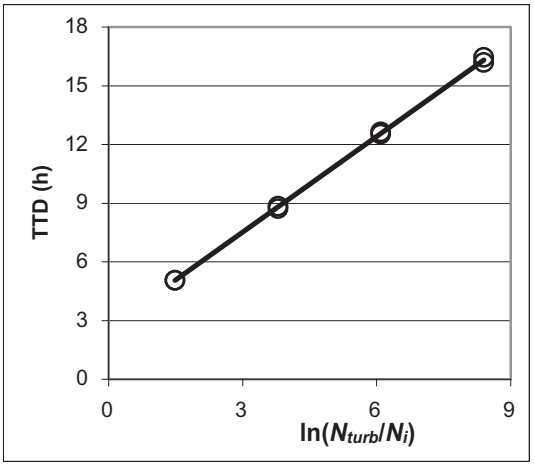

Fig. 2. Example plot of the serial dilution method (equation (1)) for *Listeria innocua* ATCC 33090 in TSBYE + 5% NaCl at 30° C. *TTD* is the time to detection, N_i is the initial cell concentration and N_{turb} the cell concentration at $abs_{600nm} = 0.2$

For the different salt and temperature combinations under study, no significant differences in μ_{max} and λ were observed for the different strains within one species. Therefore, no individual strain results are presented, but the results are grouped per species, i.e., *L. monocytogenes* and *L. innocua*. This also implies that the reported standard deviation values reflect both strain and replicate variation.

3.1. Growth rate estimations

The estimated maximum growth rates obtained by using the serial dilution method are presented in Table 2.

Table 2: Effect of temperature and salt concentration on the maximum growth rate (mean maximum growth rate, μ_{max} (h^{-1}) ± SD) of *Listeria monocytogenes* and *Listeria innocua* strains in TSBYE.

Salt (%)	Species	Temperature (ºC)		
		30	12	4
0	*Listeria monocytogenes*	1.03 ± 0.01	0.185 ± 0.004	0.042 ± 0.002
	Listeria innocua	1.05 ± 0.02	0.179 ± 0.003	0.034 ± 0.001
2.5	*Listeria monocytogenes*	0.85 ± 0.02	0.158 ± 0.006	0.032 ± 0.002
	Listeria innocua	0.89 ± 0.03	0.157 ± 0.001	0.024 ± 0.002
5	*Listeria monocytogenes*	0.49 ± 0.05	0.124 ± 0.007	0.025 ± 0.05
	Listeria innocua	0.62 ± 0.02	0.119 ± 0.007	0.010 ± 0.02

From Table 2 it can be seen that the maximum specific growth rate decreased with decreasing temperature. An approximate 80% decrease in maximum specific growth rate was observed between 30 and 12ºC. A similar decrease was observed when the temperature was further reduced to 4ºC. Table 2 also shows the effect of increasing the salt concentration from 0% to 5%. At 30ºC, this increase in salt concentration reduced the growth rate by approximately 50%. A 30% reduction in growth rate was observed at 12ºC. For *L. monocytogenes* a similar reduction (30%) was observed at 4°C. Overall *L. innocua* showed a higher growth rate than *L. monocytogenes* at 30ºC. At 12ºC the growth rates were similar for both species, while at 4ºC *L. monocytogenes* showed the higher growth rates.

3.2. Lag time estimations

The estimated lag times obtained by using the TTD method are presented in table 3.

Table 3: Effect of temperature and salt concentration on lag time (mean lag time, λ (h) ± SD) of *Listeria monocytogenes* and *Listeria innocua* strains in TSBYE.

Salt (%)	Species	Temperature (ºC)		
		30	12	4
0	*Listeria monocytogenes*	1.1 ± 0.1	5.7 ± 0.7	37 ± 7
	Listeria innocua	1.5 ± 0.5	6.8 ± 1.7	80 ± 8
2.5	*Listeria monocytogenes*	1.3 ± 0.2	6.5 ± 1.4	23 ± 11
	Listeria innocua	1.7 ± 0.3	10.0 ± 0.7	35 ± 18
5	*Listeria monocytogenes*	1.0. ± 0.5	9.9 ± 1.6	48 ± 26
	Listeria innocua	2.2 ± 0.5	15.5 ± 2.4	71 ± 24

For *L. monocytogenes* the lag time increased with decreasing temperature. From Table 3 it can be seen that the increase is approximately 5-fold from 30ºC to 12ºC and 3-8 fold between 12 and 4ºC. Salt concentration also affected the lag time of *L. monocytogenes*. At 30°C, however, the estimated lag time values were in the same range for the different salt concentrations. The increase in lag time between 0 and 5% salt was approximately 2-fold at 12ºC. While at 4ºC, an increase was observed between 0 and 5% salt, this was not reflected at 2.5%. However, for these data (2.5 and 5% salt at 4ºC) additional replicated experiments still have to be included. For *L. innocua* an increase in lag time can also be seen for decreasing temperature. The increase was approximately 5 fold from 30 to 12ºC. From 12 to 4ºC the increase was between 4 and 10-fold. At 30ºC the increase in salt concentration from 0 to 5% resulted in increase of the lag time from 1.5 to 2.2. The lag time estimate showed a 2-fold increase for the same increase in salt concentration at 12ºC. At 4ºC, collection of additional data will permit elucidation of effects of salt concentration. In general the lag time of *L. innocua* increased more than that of *L. moncytogenes* with increasing salt concentration and decreasing temperature.

4. Conclusion

The results show an increase in growth rate of *Listeria* with increasing temperatures. The levels were quite similar for both species, although *L. monocytogenes* showed the higher growth rate at 4ºC. Lag time was more affected than growth rate; both by temperature and salt concentration, and these effects were most prominent for *L. innocua*. 20 to 40 fold increase in lag time was observed when temperature was decreased from 30 to 4ºC. Increasing salt concentration from 0 to 5% approximately doubled the lag time.

The different growth responses shown by *L. innocua* and *L. monocytogenes* must lead to caution with respect to the potential use of *L. innocua* as a model organism for *L. monocytogenes* when studying the effects of storage temperature and salt concentration, and this mainly due to the likelihood of overestimating the lag time. As temperature and salt are the major factors for controlling growth of *Listeria* in salted fish products, their specific content is relevant to product development and food safety.

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